rpoB gene fragments and a method for the diagnosis and identification of Mycobacterium tuberculosis and non-tuberculosis Mycobacterial strains

FIELD OF THE INVENTION

The present invention is related to rpoB gene fragments and a method for the diagnosis and identification of Mycobacterium tuberculosis and non-tuberculosis Mycobacterial strains using rpoB gene fragments.

BACKGROUND OF THE INVENTION

Since the early 1980s, there has been an increase in disease caused by organisms called nontuberculous mycobacteria (NTM), which is the generic name for mycobacteria other than *M. tuberculosis* and *M. leprae* (MOTT). They affect both immune-competent and immune-compromised persons, and patients with the human immunodeficiency virus (HIV) are known to be especially vulnerable. The most frequent NTMs involved in disease cases are known to be *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, *M. fortuitum* complex, *M. chelonae*, *M. abscessus, M. szulgai*, *M. malmoense*, *M. marinum*, *M. ulcerans*, and *M. africanum, M. bovis* (28). Clinical diagnosis and treatment of nontuberculous mycobacterial infections are an increasingly frequent challenge to clinicians.

Currently, clinical diagnosis of mycobacteria to the species level is primarily based on cultural and biochemical tests. These conventional tests take several weeks, and the tests sometimes fail precise identification. The procedures for these tests are complex, laborious, and are usually impeded by the slow growth of mycobacteria in clinical laboratories. Additional methods, such as high-performance liquid

chromatography, gas-liquid chromatography, thin-layer chromatography (5, 21, 36), and DNA sequencing analysis (3, 4, 15, 16, 17, 19, 26, 31, 32) can differentiate mycobacteria to the species level, but these are labor-intensive and difficult to perform for routine use in many clinical laboratories.

In contrast to the above-mentioned techniques, recent molecular techniques employing PCR-amplified products offers an easy, rapid, and inexpensive way to identify several mycobacterial species in a single experiment. PCR-restriction fragment length polymorphism analysis (PRA) has been developed to target mycobacterial genes, which are present in all mycobacteria such as *hsp65* (7, 11, 25, 29, 30, 34, 35), 16S rRNA (2, 14, 37), and *dnaJ* (33). However, these techniques are still cumbersome since they require several enzyme digestions for species identification, and the results are not easy to interpret for species identification due to the limited size variation of DNA fragments after digestion.

On the other hand, probe-hybridization technique which employs DNA of the clinical specimen and oligo-probe hybridization (8, 9, 10, 18, 20, 23) is a useful tool for direct and rapid identification of NTM species. However, commercial kits currently available in the market are very expensive, limited only to 5 mycobacterial species, and the identification of a single species requires an independent experiment.

SUMMARY OF THE INVENTION

The present invention provides DNA fragments including sequence SEQ. ID. NO. 1 to 4 and 6 to 24.

The present invention provides a method of identification of Mycobacterium strain comprising the step of 1) digesting a DNA fragment which has one of the sequence Seq. ID. NO 1 to 4 to 24 with restriction enzyme to obtain DNA fragment

length polymorphism pattern ; 2) isolating DNA fragment from microorganism to identify; 3) amplifying said DNA fragment; 4) digesting said amplified DNA fragment with the same restriction enzyme in step 1) ; 5) obtaining DNA fragment length polymorphism pattern from DNA fragment in step 4) ; and 6) comparing DNA fragment length polymorphism pattern from step 1) with DNA fragment length polymorphism pattern from step 5).

Preferably, said restriction enzymes are enzyme HaeIII, MspI, Sau3A1 or BstEII.

Preferably, the DNA fragment length polymorphism pattern from steps 1) and 5) is obtained by electrophoresis.

And the Mycrobacteria strain to be identified by this method are preferably *M. tuberculosis, M. avium, M. abscessus, M. flavescence, M. africanum, M.bovis, M.chelonae, M. celatum, M. fortuitum, M.gordonae, M.gastri, M. haemophilum, M.intraecllulare, M. kansasii, M. malmoense, M. marinum, M. szulgai, M. terrae, M. scrofulaceum, M. ulcerans or M. xenopii.*

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. (A). A diagram showing amplified region of the *rpoB* for PRA in this study. The primers PRO5' and RPO3' generates 360-bp PCR product, which locates upstream of *rif*^{r} region associated with resistance of *M. tuberculosis* to rifampin.

(B). An agarose gel (2%) with 360-bp PCR products using PRO5' and RPO3'. Lane M. DNA size marker (100-bp ladder), lane 1: negative control (no DNA sample), lanes 2-11: PCR products with reference strains of mycobacteria.

Fig. 2. An example of PRA results with reference strains of mycobacteria using a set of primers (RPO5' and RPO3'). Amplified DNA was digested using both (A) *Msp* I and (B) *Hae* III restriction enzymes, and run on a 4% Metaphore agarose gel. Lane M:

DNA size marker (50-bp ladder), lane 1: *M. gordonae* type IV, lane 2: *M. szulgai*, lane 3: *M. kansasii* type I, lane 4: *M. gallinarum*, lane 5: *M. avium*, lane 6: *M. scrofulaceum*, lane 7: *M. asiaticum*, lane 8: *M. chelonae*, lane 9: *M. moriokaese*, lane 10: *M. phlei*, lane 11: *M. pulveris*, lane 12: *M. fortuitum* type I, lane 13: M. *austroafricanum*, lane 14: M. *smegmatis*, lane 15: *M. marinum*.

Fig. 3. PRA results with clinical isolates that have been identified by conventional methods, including microbiological and biochemical tests. PCR products were digested with *Msp* I enzyme and electrophresised on 4% Metaphore agarose gel. Strains were clinical isolates of (A) *M. kansasii*, (B) *M. tuberculosis*, and (C) *M. chelonae* complex that include *M. chelonae sub. chelonae* and *M. chelonae sub abscessus*.

Fig. 4. An algorithm was constructed based on the results of PRA with 40 mycobacterial reference strains and 3 other related bacterial strains. The PRA results of 10 other mycobacterial reference strains are not listed in this figure to make the algorithm concise.

Fig. 5. An example of the application of *rpoB*-based PRA for the species identification of mycobacterial clinical isolates in clinical laboratory. Clinical isolates were amplified using primers, RPO5' and RPO3', digested with *Msp* I, and run on a 4% Metaphore agarose gel. A DNA size marker (lane M: 50-bp ladder) and the PRA result of *M. bovis* was used as an internal size marker (lane 16) for each test. Using the algorithm in Fig. 3, these clinical isolates were determined to be *M. intracellulare* (lanes 1-6, 8, 9, 11-15), *M. gordonae* type II (lane 7), and *M. abscessus* (lane 10).

Fig. 6. Sequence alignment of the *rpoB* region amplified using a set of primers RPO5' and RPO3'from 35 different mycobacterial species. Sequences were aligned using multi-align program(6). Dashed lines represent nucleotide gaps.

Fig. 7. Examples of PCR-dot blot hybridization experiments. A total of 48 PCR products generated by using primers, RPO5, and RPO3', and DNAs from 48 mycobacterial species were blotted on the membrane, and an oligonucleotide probe which is specific to a certain mycobacterial species was hybridized at conditions described in the Materials and Methods section. Blotted DNAs on the membrane were as following; 1: *M. tuberculosis*, 2: *M. scrofulaceum* 3: *M. szulgai,* 4: *M. gastri*, 5: *M. kansasii* type I, 6: *M. kansasii* type II, 7. *M. kansasii* type III, 8: *M. kansasii* type IV, 9: *M. kansasii* type V, 10: *M. terrae*, 11: *M. avium*, 12: *M. intracellularae,* 13: *M. africanum*, 14: *M. celatum* type I, 15: *M. celatum* type II, 16: *M. haemophilum*, 17: *M. malmoense*, 18: *M. bovis*, 19: *M. chelonae,* 20: *M. abscessus*, 21: *M. ulcerans*, 22: *M. marinum*, 23: *M.genevanse*, 24: *M. simiane*, 25: *M. flavescens,* 26: *M. fortuitum* type I, 27: *M. fortuitum* type II, 28: *M. peregrinum,* 29: *M. triviale*, 30: *M. phlei*, 31: *M. parafortuitum*, 32: *M. vaccae,* 33: *M. aurum*, 34: *M. neoaurum*, 35: *M. fallax,* 36: *M. xenopi,* 37: *M. aichiense,* 38: *M. mucogenicum,* 39: *M. nonchromogenicum, 40*: *M. senegalense*, 41: *M. smegmatis*, 42: *M. thermoresistable*, 43: *M. intermedium,* 44: *M. gordonae* type I, 45: *M. gordonae* type IL, 46: *M. gordonae* type III, 47: *M. gordonae* type IV, 48: *M. bovis*, BCG

DETAILED DESCRIPTION OF THE INVENTION

Mycobacterial identification to the species level is not only of academic

interest but also is important because it provides a great deal of useful information on the epidemiology and pathogenesis of the organism, suggesting potential intervention strategies including successful treatment of patients on the clinical base. It is therefore important to develop methods that are rapid and simple, but yet precise and cost-effective to be used in a wide variety of clinical laboratories around the world. Currently available methods for differentiation of mycobacteria to the species level are time-consuming evaluations using phenotypic and biochemical tests or laborious procedures using expensive equipment.

As compared to other molecular methods, the PRA method certainly fits these requirements better. It is rapid and precise since it employs PCR, and simple and cost-effective since it does not require any expensive equipment or laborious processes and can differentiate numerous species of mycobacteria within a single experiment. Owing to these advantages, several PRA methods based on different genes of mycobacteria have been developed (2, 7, 11, 14, 25, 29, 30, 33, 34, 35, 37). However, most of those methods require use of more than two enzymes to differentiate mycobacteria at the species level, and require computer-assisted software program to differentiate restriction fragments since the profiles of certain mycobacterial species were not distinctive enough for bare-eye interpretation.

The new PRA method developed through this invention has more advantages that the previous ones. As presented in Fig. 1, it is apparent that most of the species harbor unique PRA profiles. Unlike other PRA profiles, which may need computer-assisted analysis and interpretation of the gels, we do not face problems in resolving all the patterns obtained during the experiments. Furthermore, problems including gel-to-gel variations or confusion with the size of the restriction fragments were limited with the use of 50-bp size marker and PRA profile of *M. bovis* as an internal size marker.

On the other hand, the four members of the *M. tuberculosis* complex that are difficult to separate by using other methods such as sequence analysis or HPLC of mycolic acids were also undistinguishable by PRA method, confirming that they do belong to a genetically similar group. However, unlike other methods, this new PRA method can further differentiate *M. africanum* from other *M. tuberculosis* complex by *Sau* 3AI digestion. Therefore, in case the clinical isolate shows the *M. tuberculosis* complex profiles, PCR products can be further processed to differentiate *M. africanum* from other *M. tuberculosis* complex by *Sau* 3AI digestion. In addition, *M. tuberculosis* and *M. bovis* can be differentiated by PCR amplification using esat-6 gene derived PCR primers, which is known to be present only in the genome of *M. tuberculosis*.

Currently in our laboratory, a substantial number of mycobacterial clinical isolates have now been identified by our new PRA method in parallel with other reference methods, including conventional tests and molecular biological methods such as PRA based on *hsp65* gene and sequence analysis based on the *rpoB* gene. As a conclusion of this experiment, it is certain that this new PRA is a rapid, cost-effective, and efficient method for the identification of mycobacteria in a clinical microbiology laboratory. The whole procedure can be done in 2 days when culture is used. PRA has been successful when using a loopful of culture taken from solid media or using 100 μl taken from liquid culture such as MGIT for mycobacterial species identification. Both of systems work well even with genomic DNA simply boiled for 5 min.

In addition to the PRA, PCR-dot blot and PCR-reverse dot blot hybridization method employing oligonucleotide probes that are highly specific to each mycobacterial species were also shown to be valuable techniques for simple and rapid identification of mycobacterial species. The oligonucleotides developed in this study were highly species-specific, thus indicating a usefulness of these probes in development of

mycobacterial identification system which can be useful in clinical settings.

To develop new molecular techniques that are easier and more precise for mycobacterial species identification than currently available ones, we chose the *rpoB* gene that encodes β subunit of RNA polymerase. The information–rich nature of the *rpoB* gene has been recently employed in differentiation of mycobacteria by DNA hybridization array (10) or by DNA sequence analysis (16). However, the *rpoB* region used in these previous studies has limited sequence variation that can be used for species identification of mycobacteria. In the present study, we extended the genetic knowledge of the *rpoB* gene to the highly polymorphic region that is suitable for developing mycobacterial species identification system using molecular biological techniques such as PRA and PCR-DNA hybridization. We also chose this region of the *rpoB* gene to be flanked by highly conserved sequences, thus can be suitable for PCR amplification of the *rpoB* region of all mycobacterial species using the same set of PCR primers.

In this study, 50 reference strains representing 44 different mycobacterial species and 6 subspecies were used to amplify the 360-bp region of the *rpoB* gene. The PCR products were then subjected to restriction fragment length polymorphism analysis (RFLP) to determine the efficacy of this region of the *rpoB* gene for mycobacterial species identification using PRA method. Subsequently, on the basis of PRA profiles generated with reference strains, an algorithm was generated, and a total of 260 clinical isolates were evaluated using new PRA method. In brief, the results clearly showed that this novel PRA method based on the *rpoB* gene generates clear and distinctive results for easy, rapid, and precise identification of mycobacterial species that can be employed in clinical laboratories for prompt and accurate diagnosis.

Subsequently, PCR amplified regions of the *rpoB* gene derived from 30

mycobacterial species that are known to have clinical importance were sequenced. In brief, results of sequence analysis showed that in the region of *rpoB* we amplified, highly polymorphic and species-specific regions exist, and thus indicated the usefulness of these regions for developing a new PCR-dot blot hybridization technique. On the basis of these sequence information, species-specific oligo-probes were designed and used to establish mycobacterial species identification system using DNA hybridization techniques such as PCR-dot blot and PCR-reverse blot hybridization method.

The restriction analysis of a 360-bp fragment within *rpoB* gene after single *Msp* I digestion is highly effective for differentiating most of mycobacteria even at the species level. Only several species require additional enzyme digestion such as *Hae* III, *Sau* 3AI, *Hinc* II, *etc.* For some species, such as *M. gordonae*, *M. kansasii*, *M. fortuitum*, and *M. celatum*, the discrimination was even obtained at the subtype level. For *M. kansasii*, this subdivision was clearly linked to genetic divergence observed previously by other investigators (1, 24, 27). It is therefore possible that using this PRA method, the discrimination at a subgroup level for other species could be similarly linked to bacteriological and clinical specificities.

Therefore, this invention provide a rpoB gene fragment(SEQ. ID. NO. 1 to 4 and 6 to 24) which has conserved sequence and polymorphic sequence between mycobacterial species.

Also this invention provide a method for diagnosis and identification of Mycobacterium tuberculosis and Non-tuberculosis Mycobacterium strain comprising the step of

1) digesting a DNA fragment which has one of the sequence Seq. ID. NO 1 to 24 with restriction enzyme to obtain DNA fragment length polymorphism pattern ;

2) isolating DNA fragment from microorganism to identify;

3) amplifying said DNA fragment using primer (SEQ. ID. NO. 25 and 26);

4) digesting said amplified DNA fragment with the same restriction enzyme in step 1) ;

5) obtaining DNA fragment length polymorphism pattern from DNA fragment in step 4) ; and

6) comparing DNA fragment length polymorphism pattern from step 1) with DNA fragment length polymorphism pattern from step 5).

Preferably, said restriction enzymes are enzyme HaeIII, MspI, Sau3A1 or BstEII.

Preferably, the DNA fragment length polymorphism pattern from steps 1) and 5) is obtained by electrophoresis.

And the Mycrobacteria strain to be identified by this method are listed in Table 1.

Though the present invention has been described with regard to its preferred embodiments, one skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the scope and spirit of the invention.

EXAMPLES

MATERIALS and METHODS

Mycobacterial samples. A total of 50 mycobacterial reference strains representing 44 mycobacterial species and 3 related species which belong to 2 different genera (Table 1) were used to develop the new PRA method in this study. Among them, 40 mycobacterial strains and 3 related species were obtained from the Korean Institute of

Tuberculosis (KIT) and the Korean National Tuberculosis Association (KNTA) in Seoul, Korea. Four species were obtained from the Korean Collection for Type Cultures (KCTC) at the Korean Research Institute of Bioscience & Biotechnology (KRIBB) and *M. abscessus,* which was recently separated from *M. chelonae* as an independent new species, was obtained from Department of Clinical Pathology at Yonsei University Medical College (YUMC). Five subtypes of *M. kansasii* were generously given by Dr. V. Vincent in the Laboratoire de Référence des Mycobactéries, Institut Pasteur in France.

Clinical isolates subjected for PRA to evaluate the new method were obtained from KIT. All clinical isolates used in this study were identified on the basis of conventional tests that include microbiological characteristics and biochemical tests. For some cases, strains were subjected for conventional PRA method based on *hsp65* gene (7, 35) to help precise identification of clinical isolates.

Table 1. Bacterial strains used in this study

	Species	Strain	Source
1	*M. abscessus*	Pettenkofer Inst.	YUMC
2	*M. africanum*	ATCC 25420	KIT
3	*M. arcinogenes*	ATCC 35753	KIT
4	*M. asiaticum*	ATCC 25276	KIT
5	*M. aurum*	ATCC 23366	KIT
6	*M. austroafricanum*	ATCC 33464	KRIBB
7	*M. avium*	ATCC 25291	KIT
8	*M. bovis*	ATCC 19210	KIT
9	*M. bovis* BCG	French Strain 1173P2	KIT
10	*M. celatum* type I/II	ATCC 51130/ATCC 51131	KIT
11	*M. chelonae*	ATCC 35749	KIT
12	*M. chitae*	ATCC 19627	KIT
13	*M. fallax*	ATCC 35219	KIT
14	*M. fortuitum* type I/II	ATCC 6841/ATCC 49404	KIT
15	*M. gallinarum*	ATCC 19710	KRIBB
16	*M. gastri*	ATCC 15754	KIT

17	*M. genavense*	ATCC 51233	KIT
18	*M. gilvum*	ATCC 43909	KIT
19	*M. gordonae*	ATCC 14470	KIT
20	*M. haemophilum*	ATCC 29548	KIT
21	*M. intracellulare*	ATCC 13950	KIT
22	*M. interjectum*	ATCC 51457	KIT
23	*M. intermedium*	ATCC 51848	KIT
24	*M. kansasii* type I-V		Pasteur Inst.
25	*M. malmoense*	ATCC 29571	KIT
26	*M. marinum*	ATCC 927	KIT
27	*M. moriokaense*	ATCC 43059	KRIBB
28	*M. mucogenicum*	ATCC 49650	KIT
29	*M. neoaurum*	ATCC 25795	KIT
30	*M. nonchromogenicum*	ATCC 19530	KIT
31	*M. parafortuitum*	ATCC 19686	KIT
32	*M. peregrinum*	ATCC 14467	KIT
33	*M. phlei*	ATCC 11758	KIT
34	*M. pulveris*	ATCC 35154	KRIBB
35	*M. scrofulaceum*	ATCC 19981	KIT
36	*M. smegmatis*	ATCC 19420	KIT
37	*M. szulgai*	ATCC 35799	KIT
38	*M. terrae*	ATCC 15755	KIT
39	*M. thermoresistibile*	ATCC 19527	KIT
40	*M. triviale*	ATCC 23292	KIT
41	*M. tuberculosis* H37Rv	ATCC 27294	KIT
42	*M. ulcerans*	ATCC 19423	KIT
43	*M. vaccae*	ATCC 15483	KIT
44	*M. xenopi*	ATCC 19250	KIT
45	*N. brasiliens*	ATCC 19296	KIT
46	*N. nova*	ATCC 21197	KIT
47	*R. equi*	ATCC 10146	KIT

DNA preparation. In order to prepare a DNA sample for PCR amplification, a loopful of bacterial colony was taken from the Löwenstein-Jensen medium and resuspended in 400 μl of distilled water in a screw-cap microcentrifuge tube. The sample was then boiled for 5 min, centrifuged for 5 min to settle down cell debris, and about 10 μl of supernatant containing

PCR amplification. The primer set used to amplify the region of the *rpoB* were 5'-TCAAGGAGAAGCGCTACGA-3'(RPO5') and 5'-GGATGTTGATCAGGGT CTGC-3' (RPO3') resulting in about 360-bp PCR product (base number 902 to 1261

and codon number 302 to 420 based on the sequence numbers for the *rpoB* gene of *M. tuberculosis* [GenBank accession No. p47766]. The primer sequences were selected from the region of the *rpoB* genes that have been previously identified from *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (12, 13, 22). The primers were made to amplify the region between the variable region and conserved region based on the genetic information for the *rpoB* gene of *Escherichia coli*. As a result, PCR products included 171-bp of variable region and 189-bp of conserved region. Variable region was amplified in this experiment based on an assumption that the polymorhpic nature of this region might help the clear distinction of each mycobacterial species using molecular biological techniques such as PRA and PCR-DNA hybridization. On the other hand, the region of the *rpoB* gene was also chosen to be flanked by highly conserved sequences, thus can be suitable for PCR amplification of the *rpoB* region of all mycobacterial species using the same set of PCR primers.

PCR was carried out in a final volume of 50 μl with 10 μl of DNA supernatant containing approximately 10 ng of genomic DNA, 10 pmole of each primers, 2 mM $MgCl_2$, 200 μM of deoxynucleotide triphosphates, and 1 unit of DyNAzyme^{TM}II DNA polymerase (FINNZYMES, Espoo, Finland). DNA samples were first denatured completely by incubation at 94°C for 5 min before amplification cycle, then amplified using a cycle that includes (1) denaturation at 94°C for 1 min, (2) primer annealing at 58°C for 1 min, and (3) elongation at 72°C for 1 min for 35 times using a Thermocycler (model 9600, Perkin Elmer). After the last amplification cycle, the samples were incubated further at 72°C for 7 min for complete elongation of the final PCR products. Positive and negative controls were always included in each PCR reaction. The positive control was the PCR mix with DNA of reference strain, *M. bovis,* and the negative control was the PCR mix without any DNA. After the PCR, the amplification results

were visualized using 1.5% agarose gel electrophoresis and ethidium bromide staining.

Restriction fragment length polymorphism analysis. After successful amplification, the 360-bp long PCR products were subjected to restriction enzyme digestion. Most of the time, 16 μl of PCR products (approximately 1 to 1.5 μg of DNA) were digested in a 20 μl of reaction volume using 5 units of *Msp* I (Boehringer Mannheim Biochemicals, Mannheim, Germany) and 2 μl of 10X reaction buffer supplied by manufacturer. Similarly, 16 μl of PCR product was digested in a 20 μl of reaction volume containing 5 units of *Hae* III enzyme (Takara Shuzo Co., LTD., Shiga, Japan) with the corresponding enzyme buffer. If necessary, additional enzyme digestions were carried out in a similar reaction condition. After 2 hours of incubation at 37° C, 4 μl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and the samples were loaded into a 4% metaphore agarose gel (FMC BioProducts, Rockland, Maine). Then, enzyme digested fragments were visualized by ethidium bromide staining and UV- light.

For the interpretation of the PRA profiles generated by each species, 50-bp ladder DNA size marker (Boehringer Mannheim, Germany) and the PRA profile of *M. bovis,* which generates about 175-bp, 80-bp, 60-bp, 40-bp restriction fragments, were used as an internal size marker. Using these size markers, the sizes of the restricted fragments of each species were determined, and an algorithm was made based on this information.

Cloning and sequence analysis. For sequence analysis, PCR products were purified by using a Geneclean kit (BIO101, Vista, Calif. USA) from an agarose gel and cloned into TOPO-TA cloning vector (Invitrogen Co., Carlsbad, CA) by the method

recommended by the manufacturer. DNA sequencing was done by the dideoxy nucleotide-chain termination method (21) using ARL automatic sequencer (Pharmacia Biotechs, Uppsala, Sweden). For each clone, M13 reverse primer and T7 promoter primer were used separately to read sequences from both directions. Sequences were aligned using a multiple sequence alignment program (6)

Oligonucleotide probes used in PCR-DNA hybridization assay Oligonucleotide probes for detecting specific mycobacterial species were designed to be 15-17 nucleotide long, and to contain 10-11 G+C content (Table 2). However, the oligonucletide probe for all the mycobacterial species (named as "Pan-TB" probe) was designed to be 20 nucleotide long. These specific oligonucleotide length and G+C content were selected, so that the hybridization conditions for each oligonucleotide to each mycobacterial DNA to be about the same.

Table 2. Oligonucleotide probes designed in this study to develop PCR-probe hybridization assay for Mycobacterial species identification

Name of oligonucleotides	Sequences of oligonucleotides	target mycobacteria
PAN-MYC	GACGTCGTCGCCACCATCGA	All mycobacterial species
TB	CATGTCGGCGAGCCC	*M. tuberculosis* complex
AVIUM	CGGTGAGCCGATCACCA	*M. avium*
INTRA	CCTGCACGCGGGCGA	*M. intracellularae*
GORDONAE	GTCGGCGATCCGATCA	*M. gordonae*
SZULGAI	TCTGAACGTCGGCGAG	*M. szulgai*
KANSASII	GGCCACGATGACCGTG	*M. kansasii*
GASTRI	TCTGAACGTCGGCGAG	*M. gastri*
FORTUITUM	CCTGAACGCCGGCCAG	*M. fortuitum*

FORTUITUM-COM	GTTCCGGTCGAGGTGG	*M. fortuitum complex*
SCROFULACEUM	CGTACGGATGGCCAGC	*M. scrofulaceum*
CHELONAE	TGGTGACTGCCACCACG	*M. chelonae*
ABSCESSUS	AGGTGACCACCACCACC	*M. abscesus*
TERRAE	GCTCAGGACGGTCAGT	*M. terrae*
ULCERANS/MARINUM	GGCCAGCCCATCACC	*M. ulcerans / M. marinum*
GENAVANSE/SIMIAE	CCAGCCGACGATGACG	*M. genavanse / M. simiae*

PCR-dot blot hybridization. To prepare the DNA dot blot, pre-cut ($10x10cm^2$) membrane (Hybond-N^+; Ammersham) was immersed into the denaturing solution (0.4N NaOH, 25 mM EDTA; pH 8.0) for 1 min. After dripping excess amount of denaturing solution, the membrane was placed on the 3MM paper, and 1-2 μl of PCR product was blotted on the membrane. Then, the membrane was air-dried for 5 min, rinsed with the denaturing solution for another 1 min, placed in-between two sheets of 3MM papers, and baked for 2 hrs at 80°C. Oligonucleotide probes were labeled by using a commercially available kit for 3'-oligolabelling and detection (ECL, Amersham Life Science). Before hybridizing with oligonucleotide probes, membrane was prehybridized at 42°C for 30 min., and subsequently hybridized with 10pmol of labeled oligonucleotide probes at 42°C for 1hr. Then, the membrane was washed twice at room temperature for 20 min, and washed twice again at 52°c for 15min. Subsequent procedures including antibody binding, washing and the signal detection were all carried out by the method recommended by the manufacturer.

PCR-reverse blot hybridization. All oligonucleotide probes to be applied on the membrane were synthesized with 5' terminal amino group, which link the oligonucleotides to the Biodyne C membrane (Pall BioSupport, East Hills, NY) by

forming covalent bond with negatively charged carboxyl group fixed on the membrane. Before blotting the oligonucleotide probes, the Biodyne C membrane was activated by incubating in 10 ml of freshly prepared 16% (w/v) 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC). After rinsed with the water, the membrane was placed on a support cushion in a clean miniblotter system (Immunetics, Inc., Cambridge, MA), and the residual water was removed from the slots. Then, the slots were filled with 150 μl of the diluted oligonucleotide solutions (approximately 200 pmol to 1 nmol of oligonucleotides in 150 μl of 500 mM $NaHCO_3$, pH 8.4). Subsequently, the membrane was incubated for 1 hr at room temperature, and then excess amount of oligonucleotide solution was removed from the slots by aspiration. In order to inactivate the membrane, the membrane was removed from the miniblotter using forceps, incubated in 100 mM NaOH for 10 min in a rolling bottle, and washed in 100 ml 2x SSPE/0.1% SDS for 5 min at 60°C in a plastic container under gentle shaking. Before applying PCR products on the Biodyne C membrane, the membrane was incubated for 5 min at room temperature in 100 ml 2x SSPE/0.1% SDS.

After placing the membrane on a support cushion into the miniblotter, in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides, residual fluid was removed from the slots by aspirations. For hybridization, about 10 μl of PCR products were diluted in 150 μl of 2x SSPE/0.1% SDS and heat-denatured for 10 min at 99°C and chilled immediately on ice. The slots were then filled with the diluted PCR products and the membrane was hybridized for 60 min at 42°C. Following hybridization, the membrane was washed in 2x SSPE/0.5% SDS for 10 min at 52°C, and incubated with 10 ml of 1:4000 diluted peroxidase labeled streptavidin conjugate in 2x SSPE/0.5% SDS for 30-60 min at 42°C in a rolling bottle. The membrane was then washed twice in 100 ml of 2x SSPE/0.5% SDS for 10 min at 42°C and rinsed twice with

100 ml of 2x SSPE for 5 min at room temperature. Finally for chemiluminiscent detection of hybridizing DNA, the membrane was incubated for 1-2 min in 20 ml ECL detection liquid and exposed to the x-ray film.

RESULTS

Since the genetic information for the *rpoB* genes of some mycobacteria are available, sequences were aligned and searched for regions, which are suitable for PRA. As a result, a set of PCR primer was selected to amplify 360-bp region of the *rpoB*, which contains polymorphic region flanked by conserved regions (Fig. 1. A.).

A total of 50 mycobacterial reference strains and 3 related bacterial strains that belong to the same Actinomycetes class with mycobacteria were used to amplify the 360-bp region of the *rpoB* gene (Table 1). The results showed the amplification of a conserved *rpoB* gene present in all mycobacteria and in some other bacteria such as *Nocardia* and *Rhodococcus* spp. (Fig. 1. B). Subsequently, PCR products were subjected to two sets of restriction enzyme digestion using *Msp* I and *Hae* III individually. These two enzymes were selected on the basis of the sequence information of the *rpoB* gene in *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (12, 13, 22).

Based on this information, PCR products were subsequently subjected for RFLP analysis (Fig. 2). In short, the result of this analysis showed that RFLP profiles of PCR products from each mycobacteria species were distinctive each other. *M. kansasii* can be easily differentiated from *M. gastri* which has much in common with non-pigmented variants of *M. kansasii*. In addition, *M. abscessus,* which has been classified as a subgroup of *M. chelonae* and was not easy to be differentiated by conventional

biochemical tests was also differentiated. Furthermore, for some species, such as *M. fortuitum*, *M. cellatum*, *M. gordonae* and *M. kansasii* that are known to contain several subtypes, each subtype generated distinctive restriction profiles. Therefore, it clearly indicated that this new PRA method could differentiate mycobacterial species at the species and even at the subspecies level.

Variable RFLP profiles generated with PCR products strongly suggested to us the polymorphic nature of this *rpoB* region amplified by PCR in this study. Then, the next question was whether these variable RFLP profiles were species-specific or also strain-specific. If strains belonging to a certain species also show polymorphic RFLP profiles, it would be too complex to use this region for the mycobacterial species identification. Therefore, clinical isolates that have been identified on the basis of conventional tests were subjected for PRA to determine the species based on an algorithm made from this study by blind tests. The results from this experiment clearly show that there is no variation among different clinical isolates that belong to the same species (Fig. 3).

On the basis of the PRA and sequence analysis results, an algorithm was constructed (Fig. 4). In an algorithm, restriction fragments smaller than 40-bp were omitted in order to reduce the confusion with primer-dimer bands. The fragment sizes are clearly separated from each other, making interpretation of results easier. In brief, the algorithm clearly shows that most mycobacterial species and other related bacterial species can be differentiated at the subspecies level by PRA using *Msp* I and *Hae* III restriction enzymes. In fact, except for several mycobacterial species, most of species can be identified by using a single enzyme, *Msp* I, thus making this new method more useful for mycobacterial species identification than previously developed PRA methods.

For those strains that are not differentiated by two enzyme digestions, the third

enzyme digestion was useful for differentiation. For example, even though the members of *M. tuberculosis* complex (*M. tuberculosis, M. bovis,* and *M. africanum*) were not differentiated by using *Msp* I and *Hae* III, the third enzyme *Sau* 3AI can differentiate *M. africanum* from other members of *M. tuberculosis* complex. In other cases, *Hinc* II can differentiate *M. gordonae* type I from *M. celatum* type I, and etc .

Subsequently, a substantial number of clinical isolates that have been identified on the basis of conventional tests were subjected for PRA (Table 3). In this experiment, a total of 260 clinical isolates were analyzed including *M. tuberculosis*, *M. avium*, *M. intracellulare, M. fortuitum, M. chelonae, M. abscessus, M. terrae M. gordonae, M. szulgai,* etc. For the easy interpretation of the PRA profiles generated by each clinical isolates, a 50-bp ladder size marker was used as a standard size marker, and the PRA profile of *M. bovis* was used as an internal size marker (Fig. 5). Results from the PRA of clinical isolates were evaluated with the help of an algorithm generated on the basis of PRA profiles of reference strains. Most of the PRA results were consistent with conventional test results, while PRA profiles of a few strains were not present in the reference algorithm. Based on the conventional tests and molecular biological sequence analysis, some of these were determined to be *"M. terrae* complex."

Table 3. Clinical isolates of mycobacteria subjected for the species identification using the new PRA.

Species Tested	No. of Clinical Isolates
M. tuberculosis	40
M. avium	40
M. intracellulare	50
M. gordonae	25
M. szulgai	10
M. fortuitum	25

M. chelonae	15
M. abscessus	15
M. kansasii	20
M. terrae	20
Total	**260**

Next, we sequenced PCR amplified region of the *rpoB* gene derived from 30 mycobacterial species that are known to have clinical importance. Subsequently, the sequences of the amplified regions were analyzed by using a software program (6). The result of the sequence analysis clearly showed that in the region of the *rpoB* we amplified, highly polymorphic regions exist, which are highly species-specific (Fig. 6). This observation suggested to us that this highly polymorphic region of the *rpoB* can be very useful to design mycobacterial species-specific oligonucleotide probes, which can be used for developing a new PCR-dot blot hybridization technique for mycobacterial species identification. Subsequently, based on the sequence information, species-specific oligonucleotide was designed (Table 3), and each oligonucleotide was used as a probe in PCR-dot blot hybridization (Fig. 7). In this experiment, a total of 48 mycobacterial species were blotted on the membrane, and each oligonucleotide was used as a probe to detect specific mycobacterial species. In brief, the results showed that each oligonucleotide probe was shown to be highly specific to each mycobacterial species targeted, indicating the usefulness of oligonucleotides for developing probe-based mycobacterial identification systems such as PCR-dot blot hybridization and PCR-reverse blot hybridization techniques.

Subsequently these probes were used to make a reverse-blot which can be used for the mycobacterial species identification system by using PCR-reverse blot hybridization method. The results showed that the PCR-reverse blot hybridization

method employing each mycobacaterial species-specific oligonucleotide probes are very efficient system for identification of mycobacteria.

All documents cited in the specification and as references below are hereby incorporated in their entirety by reference.

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